MYCOTOXINS OCCURRENCE AND FUNGAL POPULATIONS IN DIFFERENT TYPES OF SILAGES FOR DAIRY COWS IN SPAIN

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ABSTRACT

Ensiling is a common practice used worldwide to preserve crops maintaining their nutritional value during long periods of storage. Silages constitute a major component of the feed ration for dairy cows, being a potential source of mycotoxins due to the possible contamination by filamentous fungi capable of producing these toxic compounds. In this study, samples of different kinds of silages (maize, grass, alfalfa, sugar beer pulp, immature corn and ryegrass) collected from farms located in four regions of Spain, were analysed to evaluate the occurrence of aflatoxins (AFs) and *Fusarium* mycotoxins. Their lactic acid bacteria and fungal populations as well as pH and water activity were also studied. *Penicillium* (4-26%), *Geotrichum* (2-21%) and *Monascus* (0.34-3%) were the main fungi identified in all the silages examined. *Aspergillus* was found in some maize, grass and alfalfa silage samples and *Fusarium* was only identified in 0.03% of grass silage samples. The incidence of AFs was low (10% of positive samples), being detected in maize, alfalfa and immature corn silage samples. Aflatoxin B_1 (AFB_1) was only found in maize silage samples (7% of the samples). *Fusarium* mycotoxins were detected in 40% of the silage samples analysed. These toxins were present in maize, grass, alfalfa, sugar beet pulp and immature corn silage samples, being fumonisins (FBs) the most commonly detected. Maize silage was the most heavily contaminated type of silage (30 positive samples out of 44): FBs were detected in 41% of the samples, 14% presented deoxynivalenol (DON), 23% 15-acetyldeoxynivalenol (15-ADON), and 16% zearalenone (ZEN). Levels of mycotoxins detected in positive samples did not exceed the EU guidance values. The lack of relationship between *Fusarium* counts and its mycotoxin concentrations suggested that mycotoxin production possibly occurred pre-ensiling or immediately post-ensiling. Outcomes showed that mould growth and mycotoxin contamination in silages should be regularly monitored in order to minimize the exposure of dairy cows to contaminated feed.

Keywords

mycotoxins, silages, fungi, multi-mycotoxin analysis, UHPLC-FLD, HPLC-MS/MS

1. INTRODUCTION

Silages are the main forage source in diets intended for ruminants in many regions of the world (Cogan et al., 2017) representing up to 50-70% of the dry matter intake (Dunière et al., 2013). Ensiling is a method of forage preservation that allows to extend the period of storage of the crops maintaining similar nutritional value to the fresh materials (del Palacio et al., 2016). It is based on a chemical process, which occurs under anaerobic conditions and in presence of lactic acid bacteria (LAB), leading to a decrease in pH values. Some chemical or biological additives can be used to control this fermentative process although farmers usually rely on the natural LAB microbiota of the crop. By decreasing the pH level, the growth of spoilage microorganisms such as bacteria, yeasts and filamentous fungi is limited (Storm et al., 2010). Although maize and grass are the most common crops preserved by this method, many other products such as wheat, barley, alfalfa, legumes or industrial by-products like sugar beet pulp or brewer´s grains could be also used for ensiling.

There are several factors which can affect the silage quality such as a deficient preparation or compression of the silage, incorrect moisture content, leakage of rain water or insect infection leading to mould growth, loss of nutritional value and mycotoxin production. *Fusarium*, *Aspergillus*, *Penicillium*, and *Alternaria* are the most important pre-harvest genera present in silages and their occurrence might be influenced by environmental factors. The occurrence of moulds at post-harvest stage is related to improper management and storage of the silages. *Penicillium* species, mainly *P. roqueforti* and *P. paneum*, are the most usually filamentous fungi found in silages (Storm et al., 2008). *A. fumigatus* has also been isolated from silages (Shimshoni et al., 2013).

Mycotoxins are secondary metabolites of low molecular weight produced by filamentous fungi. These toxic compounds can occur in pre-harvest or post-harvest stages, and some of the main mycotoxins found in silages are aflatoxins (AFs), fumonisins (FBs), deoxynivalenol (DON), and zearalenone (ZEN) (Alonso et al., 2013). AFs are mycotoxins mainly produced by species of the genera *Aspergillus*, mainly *A. flavus* and *A. parasiticus*. These compounds are highly

toxic and can cause carcinogenic, mutagenic and teratogenic effects (Bakirdere et al., 2012). Among them, aflatoxin B_1 (AFB_1) is the most toxic, being considered as the most powerful natural hepatocarcinogenic agent in mammals. The International Agency for Research on Cancer (IARC) has classified this toxin as a Group 1 human carcinogen (IARC 2012). A chronic exposure to AFB_1 can affect to feed intake, growth rate, milk yield or to the immune system (Pereyra et al., 2008). As a consequence of the ingestion of AFB_1-contaminated feed, aflatoxin M_1 (AFM_1), an hydroxylated metabolite of AFB_1, can be excreted in milk of dairy cattle (JECFA, 2001). DON, ZEN and FBs are *Fusarium* mycotoxins. DON is a type B trichothecene produced mainly by *F. graminearum* and *F. culmorum* which inhibits protein synthesis, has immunotoxic and cytotoxic effects (Pestka, 2007), and also affects to feed intake and milk production (Rodrigues, 2014). ZEN is an oestrogenic compound produced primarily by *F. graminearum* which can cause reproductive problems. Ruminal microbiota is able to convert ZEN to its metabolites α-zearalenol (α-ZEN) and β-zearalenol (β-ZEN). Although α-ZEN has higher affinity for oestrogen receptors than ZEN, the absorption rate is lower. FBs are produced by *F. proliferatum* and *F. verticillioides* and have hepatotoxic and immunotoxic effects (Rodrigues, 2014). FB_1 and fumonisin B_2 (FB_2) have been classified as probably carcinogenic (Group 2B) by IARC (IARC, 2002, 1993) `while ZEN and DON are not considered as carcinogenic agents (Group 3) (IARC, 1993). Because of their toxicity, many countries have legislated the presence of mycotoxins in animal feed. The European Commission has set a maximum level of AFB_1 of 20.0 µg/kg in feed materials (EU, 2002) and guidance levels for the presence of DON, ZEN and FBs in products intended for animal feeding (EC, 2006).

Fusarium mycotoxins are the most common mycotoxins found in silages, especially in maize silage (Driehuis et al., 2008a, 2008b; Mansfield and Kuldau, 2007; Rodrigues, 2014). DON is the most frequently detected mycotoxin, being present at different concentrations, and it usually co-occurs with ZEN in maize silage (Driehuis et al., 2008b; Gallo et al., 2015; Whitlow and Hagler Jr., 2005). The incidence of fumonisin B_1 (FB_1) in maize silage has been reported to be

very low (Dell'Orto et al., 2015) as well as that of AFB_1, which has been rarely detected contaminating silages (Scudamore and Livesey, 1998). Other kinds of silages, as grass silage, are less contaminated by these compounds (Driehuis et al., 2008b). The most common situation observed in silages is multi-mycotoxin contamination, which is of special concern due to the potential additive or synergistic effects. In addition, another issue to take into account is the possible presence of modified mycotoxins (Cheli et al., 2013), derivatives of mycotoxins whose structure has changed due to their binding with other components of the matrix, or to the modification of their basic structure caused by chemical or biological reactions, so that they are not quantified with the usual analytical methods directed to detect the parent mycotoxins. Consequently, it is essential to develop analytical methods that allow the detection of multiple mycotoxins and their modified forms in such a complex matrix as silage.

Ruminants are considered relatively resistant to mycotoxins due to the capacity of the rumen microbiota to degrade them into less toxic compounds. However, the ingestion of fungal contaminated feed could represent a risk factor to ruminant health (Bennett and Klich, 2003; Kalac and Woolford, 1982). Besides the adverse effects on animals, mycotoxins are a Public Health concern due to the potential transfer of these compounds to animal derived-food products, such as milk or meat (Fink-Gremmels, 2008).

Studies of mycotoxins in animal feed have been usually carried out analysing the presence of these compounds in commodities such as cereals and grains (Cheli et al., 2013). Nevertheless, much less attention has been paid to the mycotoxin contamination in silages, despite of being one of the main components in ruminant diet. The aim of this work was to study the quality of silages of different botanical composition through the analysis of pH and water activity (a_w), lactic acid bacteria (LAB) and fungal populations during the availability period of each silage. In addition, the occurrence of AFs was assessed with an UHPLC-FLD method and of *Fusarium* mycotoxins with an HPLC-MS/MS multi-mycotoxin in-house validated method.

2. MATERIAL AND METHODS

2.1. Sampling

From March to August 2018, a total of 251 samples from 16 different trench silos and 3 silo bags were collected from dairy farms located in four different areas of Spain (Castilla-León, Cantabria, Galicia and Cataluña).

Samples were taken from trench and bag silos of six different botanical compositions: maize, grass, immature corn, alfalfa, ryegrass and sugar beet pulp. Samples (2-3 kg each) were collected periodically from the opening of each of the silos until the end of them (3 to 8 time points). Sampling was performed manually by removing the silages vertically from the top to the bottom of the face silo. For trench silos (maize, grass, sugar beet pulp and ryegrass silages), samples were collected at three different points, left, right and the central part (Figure 1a), having a total of 132, 78, 15 and 9 samples of these silages, respectively. With regard to silo bags (immature corn and alfalfa silages), samples were taken only from one point of the silo (Figure 1b), with a total of 10 and 7 samples, respectively.

Samples were homogenised, and subsamples of each fresh silage were randomly taken under sterile conditions for the microbiological analysis and to evaluate water activity and pH. The rest was dried at 55 °C for 24 h and ground into fine powder to be analysed for the presence of mycotoxins.

2.2. Chemical and reagents

Methanol and acetonitrile, both HPLC grade, and n-hexane were supplied by Scharlab (Sentmenat, Spain). Glacial acetic acid and ammonium formate were obtained from Fisher Scientific (Loughborough, UK). Water was purified by a Mili-Q system (Millipore, Billerica, MA, USA). Phosphate buffer saline (PBS) was prepared with potassium chloride (0.2 g) (Panreac, Castellar del Vallès, Spain), potassium dihydrogen phosphate (0.2 g) (Panreac), disodium phosphate anhydrous (1.16 g) (Panreac) and sodium chloride (8.0 g) (Panreac) in 1 L of pure water; the pH was brought to 7.4. Standard solutions of AFB_1, aflatoxin B_2 (AFB_2), aflatoxin G_1 (AFG_1) and aflatoxin G_2 (AFG_2) were purchased from Sigma (Sigma-Aldrich, Alcobendas, Spain) and standards of DON, 15-acetyldeoxynivalenol (15-ADON), 3-

144 acetyldeoxynivalenol (3-ADON), deoxynivalenol-3-glucoside (DON-3-Glc), ^{13}C-labelled DON
145 ($^{13}C_{15}$-DON), FB_1, FB_2, ZEN, α-ZEN, β-ZEN, were supplied by Biopure (Coring System
146 Diagnostix, Gernsheim, Germany). Glass microfiber filters (Whatman No. 113) were obtained
147 from Whatman (Maidstone, UK). Immunoaffinity chromatography columns (IAC) for AFB_1,
148 AFB_2, AFG_1 and AFG_2 (Easi-extract® Aflatoxin) were purchased from R-Biopharm (Rhône
149 LTD Glasgow, UK) and Multisep® 226 Aflazon+ Multifunctional columns for *Fusarium*
150 mycotoxins, from Romer Labs Diagnostic GmbH (Tulln, Austria).

151 Stock solutions of AFB_1, AFB_2, AFG_1 and AFG_2 were obtained by dissolving standard solutions
152 in methanol. Working solutions were prepared by appropriate dilution of stock solution with
153 methanol:water (50:50, v/v). For FB_1, FB_2, DON, ZEN, α-ZEN and β-ZEN stock solution were
154 made by diluting the standard solutions in methanol, and, in acetonitrile for 15-ADON, 3-
155 ADON, DON-3-Glc, $^{13}C_{15}$-DON. Working solutions were prepared using the appropriate
156 solvent. All solutions were stored at -4 °C.

157 **2.3. Water activity (a_w) and pH analysis**

158 Water activity (a_w) and pH of fresh silage samples were determined. For the pH measurement,
159 100 mL of distilled water was added to 10 g of sample. The pH was measured with a Basic20
160 PH-Meter (Hach Lange Spain SLU, Alella, Barcelona, Spain) after shaking for 15 min. The a_w
161 was determined using an AquaLab Series 3 (Decagon Devices, Inc., WA, USA).

162 **2.4. Microbiological analysis**

163 Fresh silage samples were stored at 4 °C until the microbial analysis. LAB count was carried out
164 for all kinds of silage, and in case of maize, grass, ryegrass and sugar beet pulp silage samples it
165 was used only the portion taken from the central part of the silo. LAB were enumerated on
166 Mann, Rogosa, Sharpe (MRS) agar medium (BIOKAR Diagnostics, Beauvais, France),
167 according to the official method (ISO 2009). Briefly, ten grams of fresh sample were placed in
168 90 mL of PBS and homogenised in a stomacher blender (Stomacher 400, Seward Medical,
169 London, UK). Serial decimal dilutions (from 10^{-1} to 10^{-6}) of the homogenate were prepared in

PBS and 100 µL were spread on the surface of the MRS Petri dishes. Plates were incubated under anaerobic conditions at 37 °C for 72 h. For counting, plates containing between 30 and 300 colony forming units (CFU) were considered and the results were expressed as CFU/g of sample.

For qualitative determination of moulds in maize, grass, alfalfa and ryegrass silage samples, the technique of direct plating was employed. Under aseptic conditions, 100 portions of each silage were placed onto the surface of 20 Petri dishes (5 portions per plate) in chloramphenicol glucose agar (CGA) medium (BIOKAR Diagnostics, Beauvais, France). Plates were incubated in darkness for 14 days at 25 °C. Classification into genera was performed according to the taxonomical descriptions of Pitt and Hocking (2009). Fungal infection was calculated as percentage based on the 100 portions from each silage that were plated.

2.5. Mycotoxins analysis

2.5.1. Aflatoxins

Five grams of ground silage sample were extracted with 40 mL of acetonitrile:water (90:10, v/v) and put into an ultrasonic bath for 10 min. Then, the sample was centrifuged at 4,676 *g* for 10 min. Three millilitres of the supernatant were diluted with 72 mL of PBS solution and passed through the IAC column at a flow rate of 3 mL/min. The column was washed with 20 mL of PBS at a flow rate of 5 mL/min and, finally the AFs were eluted using 1 mL of methanol and 1 mL of mili-Q water, consecutively. Sample extract was filtered through a 0.22-µm PTFE disposable syringe filter (Kinesis, Cambridge, IK) before the injection in the UHPLC system.

The detection of AFs was performed using an Agilent 1260 Infintiy Quaternary LC system (Agilent Technologies, Santa Clara, California, United States) equipped with a quaternary pump, an autosampler, a vacuum degasser and a fluorescence detector set at 365 nm and 440 nm for excitation and emission, respectively. The separation was achieved with a Poroshell 120 EC-C18 UHPLC column (2.7 µm particle size, 4.6 x 50 mm; Agilent Technologies) protected with a Poroshell 120 EC-C18 UHPLC Guard 3PK (2.7 µm particle size, 4.6 x 5 mm; Agilent

196 Technologies). The post-column derivatization to detect AFB_1 and AFG_1 was carried out with a
197 LCTech UVE photochemical system (LCTech GmbH, Obertaufkirchen, Germany). A solution
198 of acetonitrile:methanol:water (10:20:70, v/v/v) was used as the mobile phase at a flow rate of
199 1.2 mL/min. The volume of injection was 50 µL and the temperature of the column was set at
200 40 °C. Parameters of the UHPLC-FLD method are summarized in Table 1.

201 **2.5.2. *Fusarium* mycotoxins**

202 For *Fusarium* mycotoxin analysis, two grams of silage sample were placed into a 50-mL
203 polypropylene centrifuge tube and were spiked with 250 µg/kg of $^{13}C_{15}$-DON, used as internal
204 standard, and allowed to stand for 30 min. Then, thirty millilitres of acetonitrile:water:acetic
205 acid (79:20:1, v/v/v) were added. The samples were horizontally shaken for 1 h at 200 rpm and
206 centrifuged at 1008 *g* for 10 min. Fifteen millilitres of the supernatant were defatted by
207 extraction with 10 mL of n-hexane for 10 min on a rotatory shaker. Then, the samples were
208 centrifuged at 1008 *g* for 10 min and the hexane layer was removed and discarded. In order to
209 be able to detect all the mycotoxins, two different clean-up pathways were followed. To recover
210 ZEN and DON, and their metabolites, MultiSep®226 Aflazon+ Multifunctional columns were
211 used. Seven millilitres of the defatted extract were placed into a test tube and the column was
212 pushed into the tube, forcing it to flow through the material of the column. Three millilitres
213 were collected in a vial. For the recovery of FBs, 6 mL of the defatted extract were filtered
214 through a glass microfiber filter and 2 mL of the filtrate were added to the vial containing the
215 Multisep® 226 eluate. The mixture was evaporated to dryness under a nitrogen steam at 40 °C
216 and re-dissolved in 1 mL of methanol:5 mM ammonium formate in water (50:50, v/v). The final
217 extract was filtered through a 0.22-µm PTFE disposable syringe filter before to be injected in
218 the HPLC system. Method performance for DON, DON-3-Glc, 3-ADON, 15-ADON, ZEN, α-
219 ZEN, β-ZEN, FB_1 and FB_2 is summarized in Table 2.

220 HPLC-MS/MS analysis was performed with an Agilent series 1290 RRLC system (Agilent,
221 Santa Clara, CA, USA) equipped with a binary pump (G4220A) and an autosampler thermostat
222 (G1330B) coupled to an Agilent triple quadrupole mass spectrometer (6460A) with an

electrospray ionization (ESI) source (G1958-65138). Chromatographic separation was achieved using Zorbax Plus C18 (1.8 µm x 2.1 x 100 mm) column from Agilent (San Jose, CA, USA). The MassHunter software (Agilent, Santa Clara, CA, USA) was used for optimization and quantification. Gradient elution was established with a mobile phase consisting of methanol (eluent A) and 5 mM ammonium formate in water (eluent B) at a flow rate of 0.2 mL/min. The gradient elution started with a linear increase of A, from 25% to 100% during 3.75 min, maintaining these conditions up to 6.00 min. The composition of the mobile phase returned to the initial conditions in 0.5 min and maintained during 1 min, resulting in a total run time of 7.5 min. The temperature of the column was set at 25 °C, and the injection volume was 5 µL.

MS analyses were carried out using selected reaction monitoring (SRM) mode with positive and negative electrospray ionization ($ESI^{+/-}$). Source gas temperature and sheath gas temperature were set at 325 °C and 400 °C, respectively, with flows of 5 L/min and 11 L/min, respectively. Nebulizer was 45 psi. Capillary and nozzle voltage were 3500 V and 500 V, respectively. Retention time and MS/MS parameters are shown in Table 3.

2.6. Statistical analysis

Results of LAB counts were log transformed to $\log_{10}$ CFU/g prior to statistical analysis. Results were subjected to non-parametric analysis of variance (ANOVA) through Kruskal-Wallis test in order to assess the effect of time, point of sampling and kind of silage. Wilcoxon test was used for comparing the effects of the different levels of treatments on pH, a_w, LAB counts and mycotoxin levels. A Spearman correlation analysis was also carried out for mycotoxins concentrations, LAB counts and values of pH and a_w. Significance level was set up at $p < 0.05$. Analyses were conducted using JMP Pro 13.1.0. Software and Microsoft Office Excel 2016.

3. RESULTS

3.1. Water activity and pH of the samples

Results of the analysis of pH and a_w of the fresh samples are summarized in Table 4. No statistically significant differences were observed when comparing the values of pH and a_w along the time of sampling (Figures 2 to 4). The point of the silo where the samples had been

taken (laterals or central part) had no significant effect, neither. Thus mean values are shown in table 4, including sampling points and times. On the other hand, values of pH were higher in silage samples of immature corn, alfalfa and grass than in samples of sugar beet pulp, maize and ryegrass silages ($p < 0.05$). All kinds of silages presented high values of a_w (mean values from 0.937 to 0.960) and no significant differences were found among them.

3.2. Microbiological survey

By using the direct plating method in CGA, fungal genera present in maize, grass, alfalfa and ryegrass silage samples, were studied. The incidence of fungal genera present in each kind of silage is shown in Table 5. Yeasts were the microorganisms more frequently found in all the types of silages analysed. Apart from that, fifteen genera of filamentous fungi were identified from the different botanical composition silage samples. For maize silage, the most frequently genera found were *Penicillium* (13%), *Aspergillus* (4%), *Geotrichum* (3%), and *Paecilomyces* (1%). It was possible to identify *A. fumigatus* in one of the samples and, *A. flavus* in two of them. Samples of grass silages were contaminated by the greatest variety of fungi, being *Penicillium* (4%), *Mucor* (4%), *Monascus* (3%), and *Geotrichum* (2%) the most usually identified genera. With regard to alfalfa silage, samples usually presented *Penicillium* (26%), *Aspergillus* (6%), *Paecillomyces* (3%), and *Geotrichum* (3%). Lastly, *Geotrichum* (21%) and *Penicillium* (21%) usually occurred in ryegrass silage samples.

Table 6 shows the mean counts and ranges of LAB found in silages of different botanical composition. Mean values were higher for sugar beet pulp, alfalfa and immature corn silage samples but there were no significant differences among different times of sampling along time (Figures 2 to 4).

Taking into account only data of all the samples from the central part of the front silos, a correlation analysis was performed including pH, a_w and LAB as variables. A significant positive correlation between pH values and LAB count (ρ=0.51) was observed, but the remaining correlations were not significant.

3.3. Mycotoxin analyses

3.3.1. Aflatoxins

For this part of the study, the total set of samples collected (n=251) was analysed, including lateral and central parts in the case of maize, grass, sugar beet pulp and ryegrass silages. However, no significant difference was observed in the level of AF contamination according to the part of the silo where the sample had been taken. Therefore, for contaminated samples, a mean value was calculated, considering that the concentration of toxin in the negative samples was equal to the limit of detection (0.1 µg/kg for AFB_1 and AFG_1, and 0.05 µg/kg for AFB_2 and AFG_2). Moreover, the statistical analysis also showed that there was no significant difference in the level of AFs according to the time of sampling.

Eleven silage samples out of 95 were positive for at least one of the AFs. The results of the analysis are summarized in Table 7. Ryegrass silage samples were the only type of silage in which none of the mycotoxins analysed were detected. Apart from that, none of the grass silage or sugar beet pulp silage samples were found to be contaminated by AFs. On the other hand, AFs were occasionally detected in samples of maize silage (9 positive samples), alfalfa silage (1 positive sample) and immature corn silage (1 positive sample). AFB_1 was only detected contaminating maize silage and AFB_2 was not present in any kind of silage. Moreover, levels of contamination were always below EU guidance values.

3.3.2. *Fusarium* toxins

As it was observed that the level of AF contamination did not depend on the area of sampling ($p > 0.05$), *Fusarium* mycotoxins analysis was performed using only the fraction from the centre of the silo (n=95). Thirty-eight out of 95 silage samples (40%) were contaminated by at least one of the *Fusarium* mycotoxins analysed. Moreover, fifteen samples (16%) were positive for more than one mycotoxin. Results of *Fusarium* mycotoxins analysis are shown in Table 7. Among the silages of different botanical composition, mycotoxins were detected in maize silage, grass silage, immature corn silage and sugar beet pulp silage. Samples of alfalfa silage and ryegrass silage were not positive for the presence of any of the *Fusarium* mycotoxins. In addition, none

303 of the samples presented 3-ADON, α-ZEN or β-ZEN. Levels of mycotoxins detected in silage
304 positive samples did not exceed the EU guidance values.

305 Thirty out of 44 samples of maize silage presented contamination by *Fusarium* mycotoxins,
306 including DON, 15-ADON, ZEN and FBs. 41% of the samples presented FBs on an average
307 concentration of 761.24 µg/kg, being the most frequently found toxins. Only one of these
308 samples presented FB_1 and FB_2 at the same time, and this sample was contaminated with the
309 highest concentration of FBs (2565.11 µg/kg). The rest of the FBs-positive samples only
310 contained FB_1. Co-occurrence of DON and 15-ADON was observed in two samples and DON
311 and ZEN did not co-occur in any of the samples analysed.

312 Regarding immature corn silage, four samples were contaminated. FBs were not found in any of
313 the samples. It is worth saying that these four samples were simultaneously contaminated by
314 DON and ZEN, and three of them also contained 15-ADON. Moreover, all of these samples
315 came from the same farm which was located in Galicia. In addition, four out of the six DON-
316 positive maize silage samples had been also collected in this farm.

317 Grass silage presented a low level of *Fusarium* mycotoxins contamination, being present in only
318 four samples. FBs were also the most commonly detected toxins, presenting one sample FB_1
319 and FB_2, one FB_1 and the other one, FB_2. Co-occurrence with 15-ADON, the other toxin
320 detected in this silage, was only found in one sample.

321 Lastly, one sugar beet pulp silage sample was contaminated, being ZEN the only toxin detected
322 in this type of silage.

323 Taking into account only DON-positive samples (n=10), the correlation analysis pointed out a
324 negative significant correlation with ZEN (ρ=-0.85). For 15-ADON-positive samples (n=15), a
325 negative significant correlation was observed with LAB counts (ρ=-0.53). Regarding positive
326 samples for the presence of ZEN (n=11) or FBs (n=21), no significant correlations were
327 observed.

4. DISCUSSION

4.1. pH and a_w values

In this work, pH and a_w of different types of silages were measured during the length of the study in order to evaluate the magnitude of these parameters related to time during the feed-out phase. In general, mean pH values of the different samples of silages were in the range of the suggested values given for different types of silages in well preserved stage (Kung et al., 2018). No statistical differences were found in the values of pH and a_w of the silage samples during the usage period of the silo, being therefore quite stable along time.

Immature corn, alfalfa and grass silage samples presented higher values of pH than the rest of the silos analysed. For maize silage, values ranged from 3.22 to 6.96 (mean value 3.86), results comparable to those obtained by other authors (Carvalho et al., 2016; Driehuis et al., 2008b; Keller et al., 2013; Pereyra et al., 2008). The mean value of a_w of maize silages was 0.955, ranging from 0.914 to 0.991, in agreement with the results of Pereyra et al. (2008) and Keller et al. (2013). Concerning grass silage, mean pH value was 4.51, similar to other studies results (Cogan et al., 2017; Driehuis et al., 2008b) and the maximum level found was 7.68.

4.2. Fungal populations

In our study, *Penicillium* was the most frequently occurring genus in the four different botanical composition silage samples examined (4-26%). Moreover, *Geotrichum* (2-21%) and *Monascus* (0.34-3%) were also detected in samples of all types of silages. A high level of incidence of yeasts was found in all kinds of silages (56-99%).

Penicillium, *Aspergillus*, *Geotrichum* and *Paecilomyces* were the main genera present in maize silage samples. Other studies have also found *Penicillium* as one of the most prevalent genus (El-Shanawany et al., 2005; Garon et al., 2006; Keller et al., 2013; Pereyra et al., 2008; Storm et al., 2010). However, *Aspergillus* has been found in a higher incidence, being *A. fumigatus* and *A. flavus* the most commonly detected (Baliukoniene et al., 2012; Carvalho et al., 2016; El-Shanawany et al., 2005; Garon et al., 2006; Keller et al., 2013; Pereyra et al., 2008; Storm et al.,

2010). These differences could be attributable to the management practices or to the climatic conditions, as *Aspergillus* are more frequent in warm climates, and *Penicillium* in cooler climates (Cheli et al., 2013). Other fungi rarely encountered were *Rhizopus, Monascus*, *Alternaria*, *Eurotium*, and *Cladosporium*. *Monascus* and *Cladosporium* were also seldom identified by Garon et al. (2006) and Storm et al. (2010), who also found fungi of the genus *Eurotium.* Baliukoniene et al. (2012) also detected *Rhizopus* in maize silage samples.

Fusarium was never found in any of the maize silage samples examined. On the contrary, in other studies, *Fusarium* was one of the most dominant genus encountered (Baliukoniene et al., 2012; Garon et al., 2006; Keller et al., 2013; Pereyra et al., 2008) . *Fusarium* is categorised as a field fungi and is not usually able to survive the ensiling process, with the exception of some specific species such as *F. oxysporum*. (Storm et al., 2010, 2008). Moreover, another fungal genus commonly detected in several studies was *Mucor* (Baliukoniene et al., 2012; El-Shanawany et al., 2005; Garon et al., 2006; Storm et al., 2010), nevertheless, it was not found in any of the maize silage samples analysed.

Less information is available related to the fungal population in silages other than maize. Although the percentage of incidence was relatively low, the widest variety of filamentous fungi was observed in grass silage samples (Table 5). *Aspergillus* was found in much less samples than in maize silages and it was the only kind of silage in which *Fusarium* genus was detected, although the percentage of incidence was very low. Baliukoniene et al. (2012) and O'Brien et al. (2005) also found these moulds, except for *Monascus*, in grass silage samples.

The variety of fungi identified in ryegrass silage samples was lower than in the rest of silage samples. Baliukoniene et al. (2012) also analysed ryegrass silage samples which presented a higher occurrence of fungi. Besides *Penicillium* and *Geotrichum*, they found more fungal genera including *Fusarium* and *Aspergillus*.

As far as LAB were concerned, mean counts were higher in alfalfa, immature corn and sugar beet pulp silages than in the rest of the samples (Table 6). Mean values were similar for maize

and grass silages, the maximum value obtained for a grass silage sample being higher than that for a maize silage sample. On the contrary, Cogan et al. (2017) observed that mean count of LAB tended to be lower in grass silages than in maize and other kinds of silages. As it has been pointed out, the correlation between pH values and LAB counts was positive and significant (ρ=0.51), an unexpected result as during ensiling LAB promote a natural fermentation that should lead to a decrease of the pH level (Scudamore and Livesey, 1998).

4.3. Mycotoxin analysis

Maize silage was the most mycotoxin contaminated kind of silage, AFB_1, AFG_1, AFG_2, DON, 15-ADON, ZEN and FBs being detected in these samples. This could be explained by its composition: maize crops have a higher content of protein and polysaccharides which can help growth and survival of fungi and other pathogens (Zachariasova et al., 2014).

4.3.1. Aflatoxins occurrence

Our outcomes showed that the incidence of AFs in the different types of silages analysed was low, being 10% of the 95 samples positive for the presence of some of these toxins.

Despite the low level of occurrence, maize silage was the most AF-contaminated type of silage (Table 7). It has been stated that the incidence and level of AFs are relatively low in comparison with other mycotoxin in well-preserved silages (Ogunade et al., 2018). In addition, the incidence of AFs in silages in Europe has been rarely reported (Panasiuk et al., 2019), since their presence is usually associated with geographical regions with tropical or sub-tropical climate (Driehuis et al., 2008b).

A high number of researches have pointed out the absence of AFs in maize silage samples (Dagnac et al., 2016; Driehuis et al., 2008b; Dzuman et al., 2014; R. Kosicki et al., 2016; Panasiuk et al., 2019; Van Pamel et al., 2011; Zachariasova et al., 2014). On the other hand, in agreement with our results, several authors have detected AFs in a low number of maize silage samples and in low concentrations (Garon et al., 2006; Keller et al., 2013; Richard et al., 2009;

405 Schmidt et al., 2015). Moreover, in some studies, higher levels of AFs have been detected in
406 maize silage (Pereyra et al., 2008; Sultana et al., 2013).

407 *Aspergillus flavus* and *A. parasiticus* are the two *Aspergillus* species primarily responsible for
408 AF production. Looking at the results of the mycological study, some samples presented
409 *Aspergillus* but AFs were not detected, since mycotoxin production is affected by several
410 factors such as moisture, relative humidity or the time course of the fungal growth (Sultana et
411 al., 2013). On the other hand, AFs could be detected in the samples, without isolating the fungi
412 that produce them. *Aspergillus* was only present in one out of the three AFB_1-positive samples.
413 One of the samples in which *A. flavus* was detected was AFG_2-positive and in one of the
414 samples positive for the presence of AFG_1, *Aspergillus* was identified. Regarding alfalfa silage,
415 AFG_1 and AFG_2 were detected in the same sample, and the mycological survey revealed the
416 presence of *Aspergillus* in this sample. Although *A. flavus* and *A. parasiticus* are the main AFs
417 producers, it is worth saying that these compounds are also synthesized by several species of
418 other genera such as *Emericella* (Varga et al., 2009).

419 **4.3.2. *Fusarium* mycotoxins occurrence**

420 The results of the present study showed a relatively low incidence of *Fusarium* mycotoxins in
421 the different kinds of silage samples analysed by HPLC-MS/MS. 40% of the samples were
422 contaminated by at least one of these toxins, and co-occurrence of more than one was found in
423 16% of the samples.

424 Maize silage samples were more frequently contaminated than the other ensiled forages. In fact,
425 the occurrence in these samples was high, being 68% of the maize silage samples positive for
426 the presence of some of these toxins. FBs were the most frequently toxins detected (41%) in an
427 average concentration of 761 µg/kg, ranging from 469 to 2565 µg/kg. Similar values were found
428 by Schmidt et al. (2015), who reported a mean FB_1 concentration of 369 µg/kg in a range
429 between 124 and 2310 µg/kg. Panasiuk et al. (2019) reported an incidence of 37% of FB_1-
430 positive samples, but with lower values of toxin, being 73.5 µg/kg the mean and 379 µg/kg the

431 highest concentration found. However, these toxins are usually detected in silage samples less
432 frequently than DON and ZEN (Driehuis et al., 2008b; Panasiuk et al., 2019; Zachariasova et
433 al., 2014).

434 15-ADON, ZEN and DON were also detected in maize silage samples (Table 7). Contrary to
435 our results, DON has been reported to be the most commonly detected mycotoxin in ensiled
436 forages and it could be found in higher concentrations (Gallo et al., 2015; Storm et al., 2008).
437 Driehuis et al. (2008b) reported a mean concentration of DON of 854 µg/kg and a maximum
438 concentration of 3142 µg/kg. In Poland, Kosicki et al. (2016) and Panasiuk et al. (2019) also
439 detected high levels of DON, with mean values of 633 and 447 µg/kg and maximum levels of
440 7860 and 4347 µg/kg, respectively. Cogan et al. (2017) in Ireland, detected DON in maize
441 silages in an average concentration of 603 µg/kg and 7111 µg/kg as the maximum
442 concentration. ZEN has been also usually detected in maize silage. This toxin, as in our results,
443 used to be present in lower concentrations than DON. Driehuis et al. (2008b) and Cogan et al.
444 (2017) detected higher mean values, 174 and 209 µg/kg, respectively. On the other hand,
445 Kosicki et al. (2016) and Panasiuk et al. (2019) reported mean values similar to ours, 69.38 and
446 82.4 µg/kg, respectively.

447 In addition, the co-occurrence of DON and ZEN has been widely reported (Driehuis et al.,
448 2008b), nevertheless, in the present study DON and ZEN did not co-occur in any of the maize
449 silage samples analysed. DON and 15-ADON were simultaneously found in only two samples,
450 and none of the other metabolites of DON or ZEN were detected.

451 In the literature, very few information about the incidence of mycotoxins in silages other than
452 maize is available, although some studies have been conducted analysing grass silage samples.
453 In the grass silage samples analysed, only 15-ADON and FBs were detected. Panasiuk et al.
454 (2019) reported that 20% of the grass silage samples analysed contained FB_1 in an average
455 concentration of 7.24 µg/kg, being 10.4 µg/kg the maximum concentration found. These values
456 were lower than the results obtained in our study (mean of 604 µg/kg and maximum of 911
457 µg/kg). However, DON and ZEN were not found in any of the grass silage samples analysed.

458 Conversely, Driehuis et al. (2008b) detected ZEN in 6% of the surveyed grass silage samples.
459 McElhinney et al. (2016) also detected ZEN, and Baliukoniene et al. (2012) and Panasiuk et al.
460 (2019), detected DON as well as ZEN in grass silage samples. Our results also confirmed that
461 the occurrence of mycotoxins is higher in maize than in grass samples, since *Fusarium*
462 mycotoxins are frequently found contaminating cereals (Placinta et al. 1999) and also probably
463 because its higher content of nutrients. Moreover, it has been observed that the conditions in
464 mould infected grass do not favour DON production and also, that the fungal species that infect
465 grass are able to produce ZEN but not DON (Driehuis et al., 2008b).

466 It was remarkable the incidence of DON, ZEN and 15-ADON found in immature corn silage
467 samples collected in a farm where also DON-positive maize silage samples were collected. This
468 specific farm was located in Galicia, a Spanish region where the weather is characterised by
469 high humidity and non-extreme temperatures. Thus, environmental conditions could have
470 affected the level of contamination, due to the fact that DON and ZEN production has been
471 associated with high-humidity conditions and moderate temperatures (Ogunade et al., 2018). In
472 addition, it was noticed that these samples presented a mean pH value of 6.6, and this may
473 indicate that the silo was not correctly preserved (Kung et al., 2018).

474 The occurrence of *Fusarium* mycotoxins in the silages analysed was not related with the results
475 of the mycological survey, since *Fusarium* fungi were only detected in an extremely low
476 percentage of grass silage samples (0.03%). It is known that *Fusarium* is frequently found
477 contaminating maize in the field (Placinta et al., 1999). However, as it has been mentioned,
478 *Fusarium* is not usually capable to survive to the ensiling process, but mycotoxins are not
479 affected by ensiling (Lepom et al., 1990, 1988). The scarce detection of *Fusarium* also indicated
480 that the risk of post-harvest contamination by these fungi is very low. Therefore, the mycotoxins
481 detected were probably produced by fungi previously present in the crop either pre-ensiling or
482 immediately post-ensiling. This lack of correlation could have been avoided if molecular tools
483 would have been applied to detect non-viable fungi. Moreover, the medium used for the
484 mycological analyses was not selective for *Fusarium* isolation, consequently, the proliferation

485 of other fungal strains and yeasts might have inhibited its growth (Garon et al., 2006; Storm et
486 al., 2010).

487 5. CONCLUSION

488 The outcomes of this study pointed out that silages are potential sources of mycotoxins despite
489 the individual mycotoxins levels were substantially lower than the EU directive or guideline
490 values. The contamination by *Fusarium* mycotoxins possibly occurred pre-harvest, based on the
491 low percentage of incidence of *Fusarium* genus identified in the mycological survey. The high
492 percentage of *Penicillium* found might be considered as a potential risk due to the wide range of
493 toxic compounds that these moulds are able to produce (Liu et al., 2003). Moreover, in this kind
494 of materials more than one mycotoxin usually co-occur which may have additive or synergistic
495 effects (Cheli et al., 2013). For these reasons, and, taking into account that silages are locally
496 made in the dairy farms from annual crops, thus mycotoxin content may be different from year
497 to year, the mould growth and mycotoxin content in silages should be regularly monitored in
498 order to assess their quality.

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503 Conflict of interest

504 The authors declare no conflict of interest.

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Figure captions

Fig. 1 Example of a trench silo (grass silage) (a) in which samples were taken from the laterals and the central part, and example of a silo bag (immature corn silage) (b) in which samples were taken only from one point of the silo

Fig. 2 pH, water activity and lactic acid bacteria counts (log_{10} CFU/g) of the maize silage samples collected along the length of the study. The y-axis represents, on the left, the scale for pH and lactic acid bacteria counts and, on the right, the scale for water activity values

Fig. 3 pH, water activity and lactic acid bacteria counts (log_{10} CFU/g) of the grass silage samples collected along the length of the study. The y-axis represents, on the left, the scale for pH and lactic acid bacteria counts and, on the right, the scale for water activity values

Fig. 4 pH, water activity and lactic acid bacteria counts (log_{10} CFU/g) of the immature corn, alfalfa, sugar beet pulp and ryegrass silage samples collected along the length of the study. The y-axis represents, on the left, the scale for pH and lactic acid bacteria counts and, on the right, the scale for water activity values

686 **Table 1.** Method parameters for AFB_1, AFB_2, AFG_1 and AFG_2 determination in silages with
687 UHPLC-FLD.

Mycotoxin	LOD[a] (µg/kg)	N	Spiking level (µg/kg)	Recovery[b] (%)	RSDr[c] (%)
AFB_1	0.1	5	2	97.05±6.66	6.86
		5	20	81.07±1.45	1.79
		5	50	79.25±1.17	1.48
AFB_2	0.05	5	2	97.36±2.90	2.97
		5	20	92.04±1.88	2.05
		5	50	90.03±1.51	1.68
AFG_1	0.1	5	2	88.36±19.55	22.13
		5	20	73.05±2.04	2.80
		5	50	70.35±3.32	4.72
AFG_2	0.05	5	2	76.22±5.66	7.43
		5	20	80.96±2.72	3.36
		5	50	81.40±1.24	1.52

688 [a]LOD = limit of detection
689 [b]Mean value ± standard deviation
690 [c]RSDr = relative standard deviation

691 **Table 2.** Method parameters for DON, DON-3Glc, 3-ADON, 15-ADON, ZEN, α-ZEN, β-ZEN,
692 FB_1 and FB_2 determination in silages using acetonitrile (79%)/water (19%)/acetic acid (1%) as
693 extraction solution and quantification with HPLC-MS/MS.

Mycotoxin	LOD[a] (µg/kg)	LOQ[b] (µg/kg)	N	Spiking level (µg/kg)	Recovery[c] (%)	RSDr[d] (%)
DON	0.75	1.0	3	500	103.62±10.27	9.91
			5	2000	129.35±34.84	26.93
			3	5000	103.39±8.27	8.00
DON-3Glc	0.75	1.0	3	100	92.69±8.04	8.68
			5	400	112.37±28.63	25.48
			3	1000	110.78±5.62	5.07
3-ADON	0.3	0.5	3	100	68.36±6.60	9.66
			5	400	98.76±6.57	6.65
			3	1000	96.49±2.21	2.29
15-ADON	0.3	0.5	3	100	102.61±8.73	8.50
			5	400	82.74±15.84	19.14
			3	1000	74.17±3.74	5.04
ZEN	0.25	0.5	3	20	104.29±6.26	6.00
			5	100	111.55±12.59	11.28
			3	500	80.77±6.49	8.04
α-ZEN	0.25	0.5	3	50	91.68±12.65	13.80
			5	100	96.68±11.73	12.14
			3	250	87.56±4.05	4.62
β-ZEN	0.25	0.5	3	50	72.02±7.87	10.92
			5	100	96.68±11.73	12.14
			3	250	87.56±4.05	4.62
FB_1	30	45	3	1000	71.97±3.41	4.74
			5	2000	76.44±3.42	4.47
			3	5000	97.49±7.60	7.80
FB_2	30	45	3	500	96.18±4.68	4.86
			5	1000	110.70±6.94	6.27
			3	2500	119.05±7.00	5.88

694 [a]LOD = limit of detection
695 [b]LOQ = limit of quantification
696 [c]Mean value ± standard deviation
697 [d]RSDr = relative standard deviation

698 **Table 3.** HPLC-MS/MS conditions.

Analyte	Retention time (min)	Ionization	Precursor ion *m/z* (fragmentor voltaje (V))	Product ion *m/z* (collision energy (V))
DON-3Glc	2.7	ESI^-	476.2 (85)	**297.1 (5)**[a]
				248.9 (25)
				203.1 (25)
DON	2.8	ESI^+	297.2 (65)	231.1 (15)
				203.1 (15)
				174.9 (25)
$^{13}C_{15}$-DON	2.9	ESI^+	312.2 (125)	**263.1 (5)**
				245.0 (5)
				216.2 (15)
				186.0 (15)
15-ADON	3.7	ESI^+	339.2 (125)	**321.1 (5)**
				261.1 (5)
				137.0 (5)
3-ADON	3.7	ESI^+	339.2 (105)	**231.1 (5)**
				203.0 (15)
				175.1 (25)
FB_1	4.2	ESI^+	722.4 (175)	352.1 (30)
				334.1 (40)
FB_2	4.7	ESI^+	706.5 (125)	**336.4 (35)**
				318.4 (45)
β-ZEN	4.8	ESI^-	323.2 (125)	174.0 (40)
				160.0 (40)
				129.9 (40)
α-ZEN	5.0	ESI^-	323.2 (125)	174.0 (40)
				160.0 (40)
				129.9 (40)
ZEN	5.0	ESI^+	319.2 (85)	**301.1** (5)
				283.1 (15)
				157.0 (35)
$^{13}C_{18}$- ZEN	5.0	ESI^+	337.3 (65)	**319.2 (5)**

	301.1 (5)
	215.0 (25)
	199.0 (15)

[a]Transition in bold was used for quantification.

700 **Table 4.** Means, standard deviations and ranges of pH and a_w of the different types of silages
701 (n=251).

	pH		Water activity (a_w)	
	Mean ± SD	Range	Mean ± SD	Range
Maize silage (n=132)	3.86 ± 0.65 [b]	3.22-6.96	0.955 ± 0.02 [a]	0.914-0.991
Grass silage (n=78)	4.51 ± 0.99 [a]	3.45-7.68	0.940 ± 0.03 [a]	0.853-0.997
Alfalfa silage (n=7)	4.82 ± 0.49 [a]	4.27-5.76	0.937 ± 0.01 [a]	0.921-0.959
Immature corn silage (n=10)	4.96 ± 1.79 [a]	3.72-8.42	0.945 ± 0.02 [a]	0.910-0.994
Sugar beet pulp silage (n=15)	3.80 ± 0.14 [b]	3.51-4.04	0.960 ± 0.02 [a]	0.937-0.992
Ryegrass silage (n=9)	4.09 ± 0.97 [b]	3.49-6.79	0.939 ± 0.01 [a]	0.917-0.955

702 *In each column, mean values followed by different letters are significantly different ($p < 0.05$).

703 **Table 5.** Fungal occurrence (%) in silage samples of different botanical composition.

Fungal genera	Occurrence (%)			
	Maize silage (n=44)	Grass silage (n=26)	Alfalfa silage (n=7)	Ryegrass silage (n=3)
Yeasts	81.02	55.91	72.86	99.67
Penicillium	12.76	4.23	26.14	21.00
Geotrichum	3.13	1.51	3.14	21.33
Rhizopus	0.36	0.17	0.29	-
Aspergillus	4.00	0.40	6.14	-
Monascus	0.34	2.86	0.14	0.33
Alternaria	0.30	0.48	-	-
Cladosporium	0.20	0.31	0.29	-
Eurotium	0.25	1.17	0.57	-
Nigrospora	-	0.04	-	-
Paecilomyces	1.05	0.22	3.29	-
Mucor	-	3.97	-	-
Chaetomium	-	0.03	-	-
Fusarium	-	0.03	-	-
Epicoccum	-	0.08	-	-
Curvularia	-	0.04	-	-
Other (sterile mycelium)	1.10	6.34	5.29	0.33

704

705

706 **Table 6.** Means, standard deviation and ranges of lactic acid bacteria counts (log_{10} colony
707 forming units/g fresh weight) (n=95).

	Lactic acid bacteria (log_{10} CFU/g)	
	Mean ± SD	Range
Maize silage (n=44)	4.60 ± 1.89	2.00-8.28
Grass silage (n=26)	4.89 ± 2.20	2.00-9.32
Alfalfa silage (n=7)	6.98 ± 0.83	5.23-8.04
Immature corn silage (n=10)	6.91 ± 0.93	4.91-8.25
Sugar beet pulp silage (n=5)	8.07 ± 0.56	7.04-8.65
Ryegrass silage (n=3)	4.51 ± 1.23	3.46-6.23

708

709 **Table 7.** Mycotoxin contamination of silage samples (n=95).

Type of silage	Mycotoxin	No. of positive samples (%)	Mean (µg/kg)	Range (µg/kg)
Maize silage (n=44)	AFB_1	3 (6.82)	0.31	0.17-0.53
	AFG_1	2 (4.55)	0.34	0.16-0.52
	AFG_2	5 (11.41)	0.18	0.10-0.29
	DON	6 (13.64)	337.72	231.05-451.53
	15-ADON	10 (22.73)	3.90	2.44-6.58
	ZEN	7 (15.91)	69.79	28.73-109.28
	FB_1+FB_2	18 (40.91)	761.24	468.95-2565.11
Grass silage (n=26)	15-ADON	2 (7.69)	8.16	7.19-9.14
	FB_1+FB_2	3 (11.54)	603.61	379.40-910.58
Immature corn silage (n=10)	AFG_2	1 (10.00)	0.12	0.12
	DON	4 (40.00)	170.08	141.33-203.89
	15-ADON	3 (30.00)	2.72	2.44-3.29
	ZEN	4 (40.00)	93.66	56.82-118.85
Alfalfa silage (n=7)	AFG_1	1 (14.29)	2.21	2.21
	AFG_2	1 (14.29)	0.91	0.91
Sugar beet pulp silage (n=5)	ZEN	1 (20.00)	50.51	50.51

710 *Mycotoxins: aflatoxin B_1 (AFB_1), aflatoxin G_1 (AFG_1), aflatoxin G_2 (AFG_2), deoxynivalenol (DON),
711 15-acetyldeoxynivalenol (15-ADON), zearalenone (ZEN), fumonisin B_1 (FB_1), fumonisin B_2 (FB_2).

Figure 1

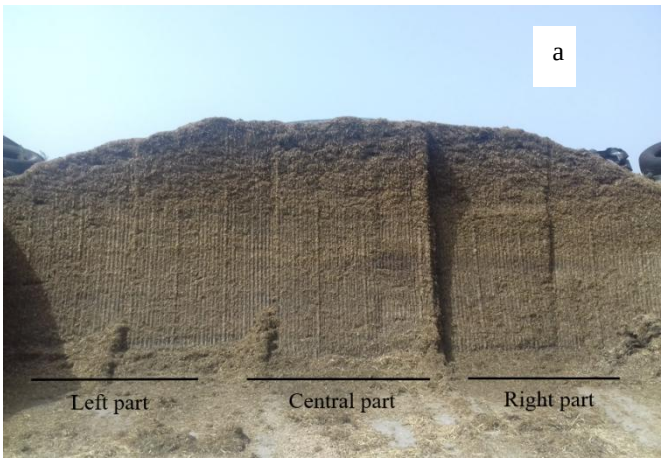
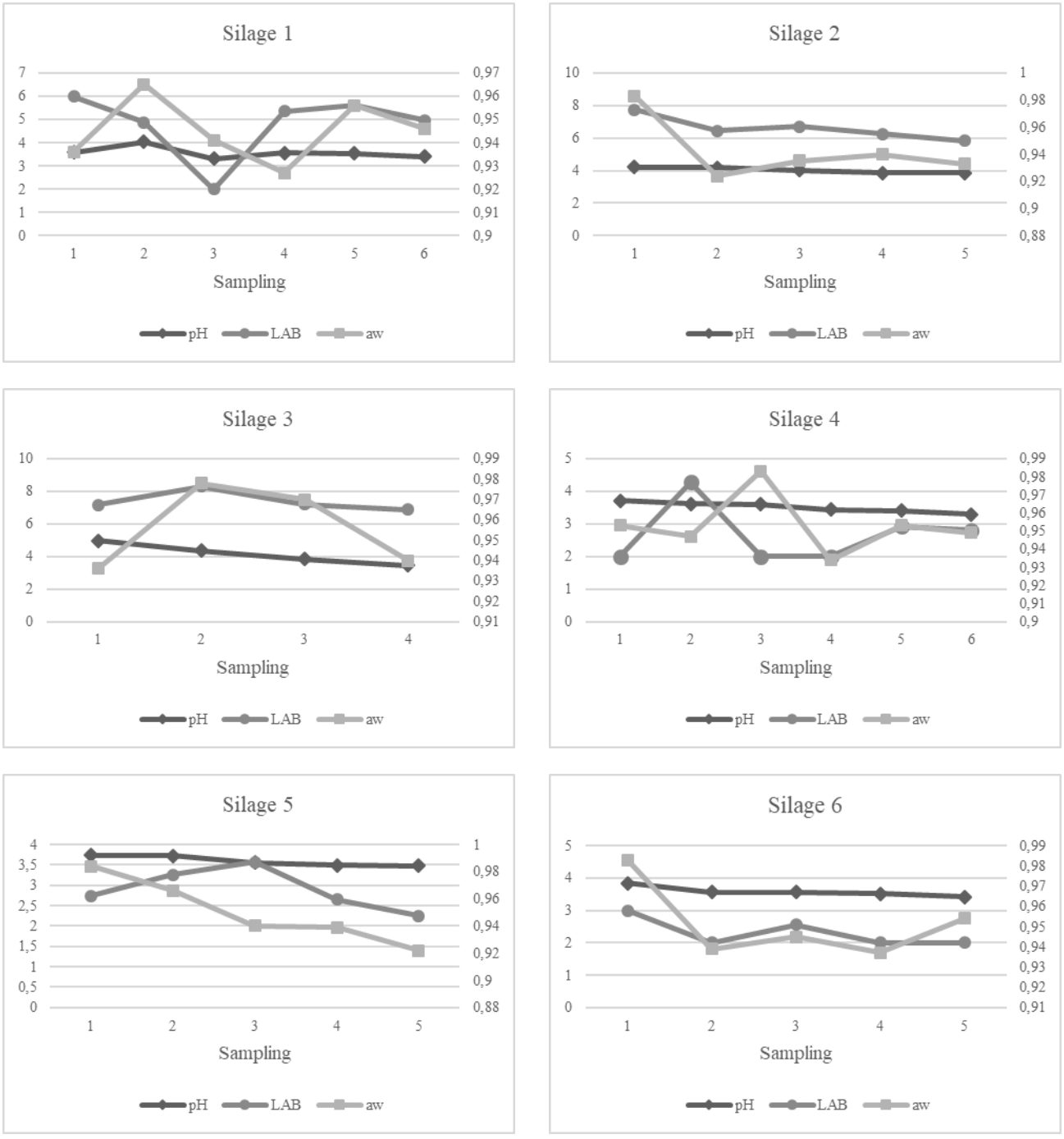


Figure 1

Figure 2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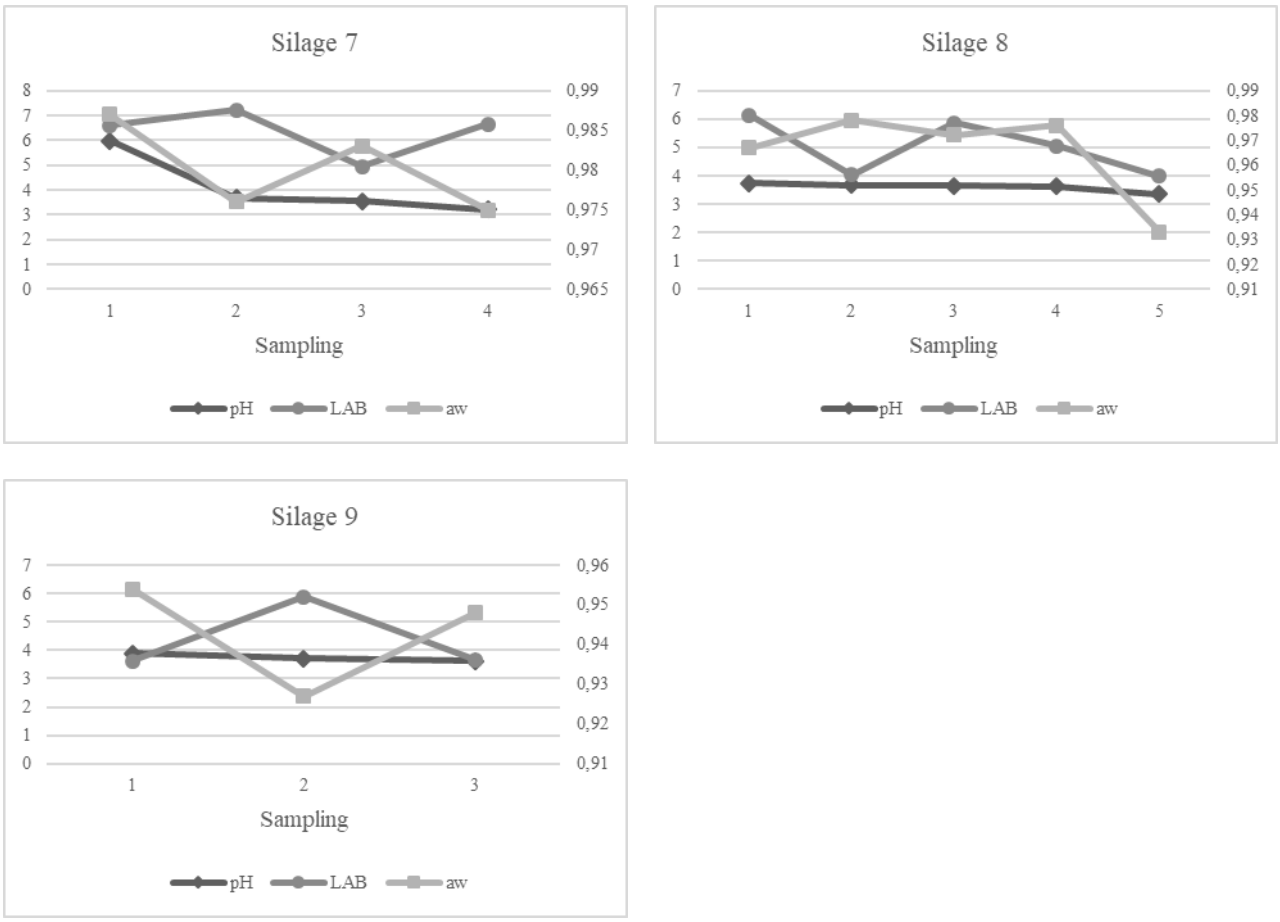
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Figure 2

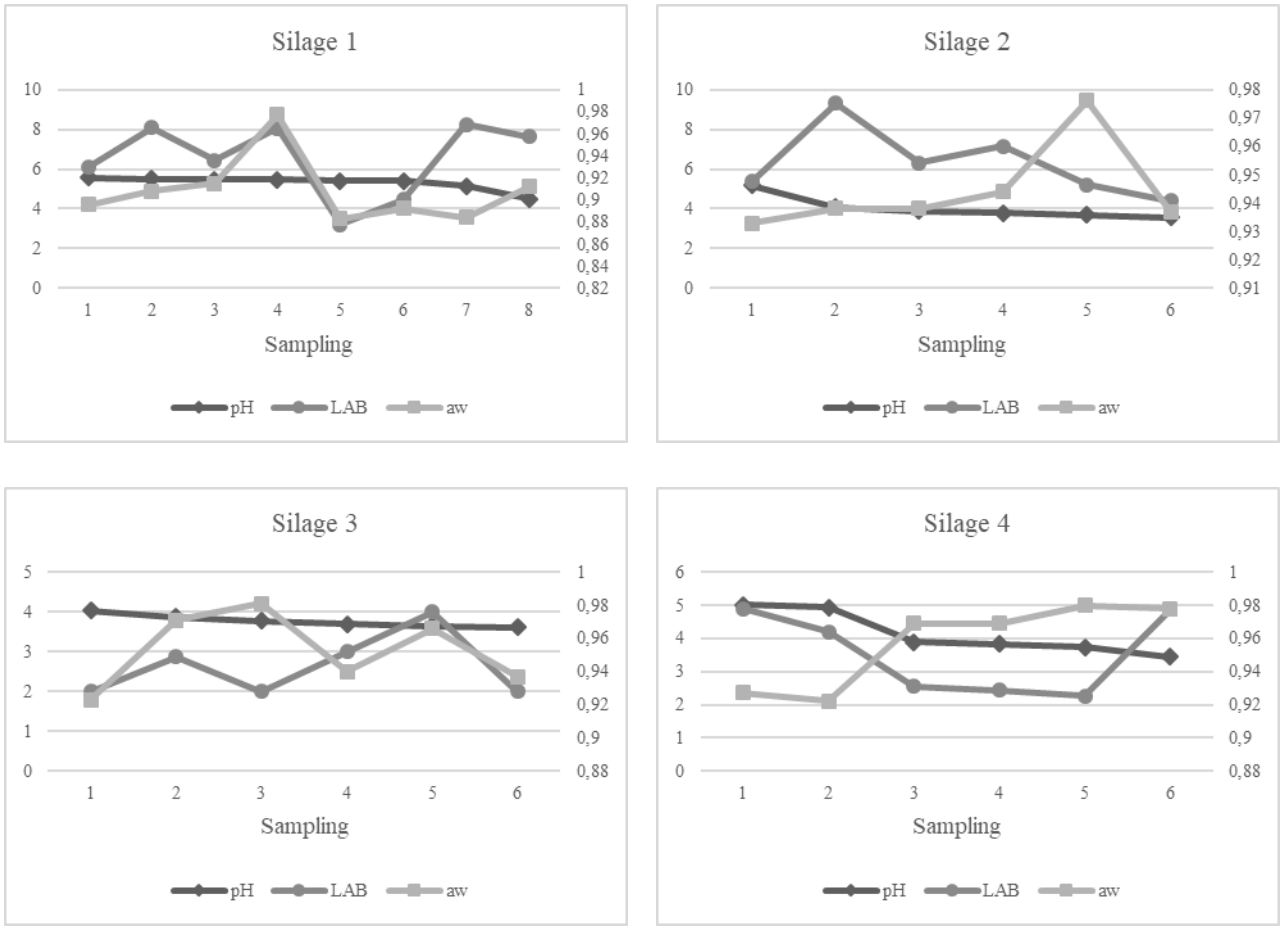
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Figure 3

Figure 4

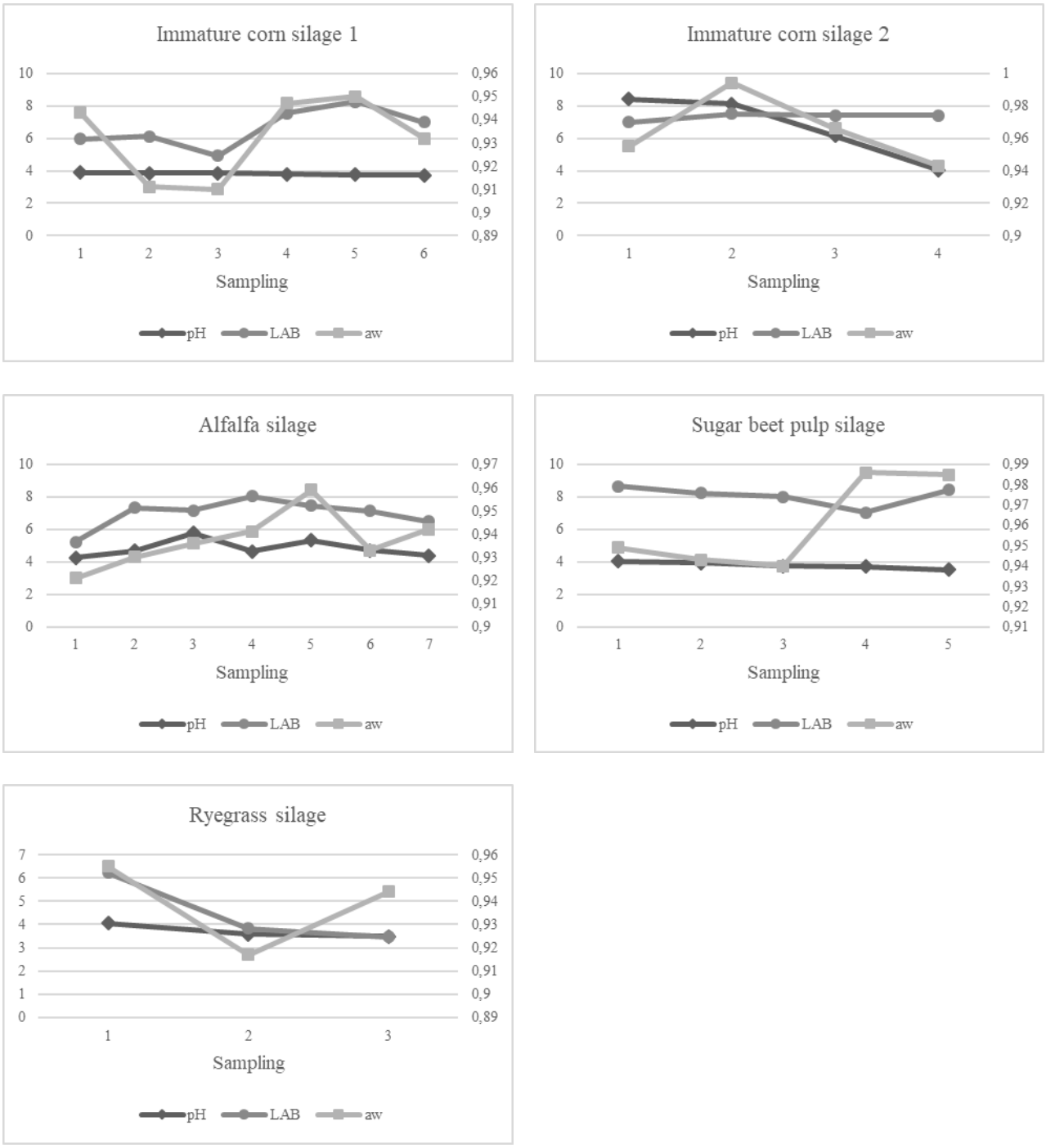

Figure 4